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REPORT ON THE CHAMBERS MODIFICATION OF  
THE WEBER AND BLACK TEST

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A number of collaborative studies have been made on the Chambers Modification<sup>1/</sup> of the Weber and Black Test<sup>2/</sup> for determining the efficiency of sanitizers and detergent-sanitizers recommended for use on dishes, utensils, and equipment in restaurants, dairies and food plants.

This method was specified in the Revision of Appendix F, Bactericidal Treatment, Milk Ordinance and Code - 1953 Recommendations of The Public Health Service issued on April 5, 1956<sup>3/</sup> for evaluating the efficiency of chemical compounds recommended for the treatment of milk equipment without direct comparison to chlorine. It has also been specified by the Department of Agriculture in Interpretation 21, Part 362 Regulations for the Enforcement of the Federal Insecticide, Fungicide, and Rodenticide Act<sup>4/</sup> for use in determining the limiting effects of water hardness on sanitizers and disinfectants recommended for these purposes. Therefore, the method does have some official status from the standpoint of law enforcement at the present time. The studies reported here were initiated to develop sufficient collaborative data so that the method could be given consideration by the A.O.A.C. and to evaluate the specific steps in the present procedure with a view to improving its precision.



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In the first study, one sample from a commercial shipment of a 10% alkyl ( $C_8H_{17}-C_{18}H_{37}$ ) dimethyl benzyl ammonium chloride was tested by four laboratories. All collaborators were instructed to adhere to the method as outlined by Chambers<sup>1/</sup> to employ synthetic hard waters as specified in Interpretation 21 and to make all tests using a solution containing 200 p.p.m. of the quaternary identified as sample X. The results of this study are summarized in Table 1. Insofar as the 30 sec. end point is concerned, this study indicated that sample X had a maximum hard water tolerance of 600 p.p.m.  $\pm$  67. The end points at the 60 and 120 second periods were considerably higher and in most instances were not actually determined.



TABLE 1. RESULTS OF UNKNOWN QUATERNARY SAMPLE X.

Laboratory	No. of Tests	Maximum water hardness in p.p.m. showing 99.999% kill of E. coli within		
		<u>30 Sec.</u>	<u>60 Sec.</u>	<u>120 Sec.</u>
J	1	500	550	> 600
	2	500	550	700
	3	500	600	> 600
K	1	600	800	> 800
	2	700	800	> 800
L	1	550	650	700
	2	550	650	700
M	1	600	> 800	> 800
	2	600	> 800	> 800
	3	600	> 800	> 800
	4	700	> 800	> 800
	5	800	> 800	> 800
12		600 $\pm$ 67		



A study of the data and statements made by the collaborators in this study indicated that the various participating laboratories had in a number of instances interpreted the Chambers directions differently, and it was believed that the resulting variations in the details of the technique were probably responsible for most of the variations in the results reported.

Therefore, a second study was initiated in which the Chambers technique was rewritten with the objective of eliminating some of the options as to details of manipulations and also amended with certain instructions to assist the collaborating laboratories in standardizing test culture suspensions. For this study, two quaternaries not currently sold for use in the field of environmental sanitation were selected as unknowns. These were relatively pure chemicals which in preliminary trials in individual laboratories had given very reproducible results and could be made available to testing laboratories as standard reference samples for use as controls on the method itself. As in the first study, all tests were made at concentrations providing 200 p.p.m. of quaternary. The two samples were tested by 8 laboratories. The results are summarized in Tables 2 and 3. The results indicate a maximum average hard water tolerance of  $404 \pm 39$  p.p.m. for unknown samples A and  $735 \pm 65$  p.p.m. for unknown sample B. From the study of individual test results in both the first and second study, it would appear that the precision of the method is probably  $\pm 10\%$ . That is, a product having a hard water tolerance of 500 p.p.m. of hardness could be expected to have a standard deviation of  $\pm 50$  p.p.m., and one with a maximum water hardness of 1000



p.p.m. a standard deviation of  $\pm 100$  p.p.m. On the other hand, the results found within individual laboratories indicate that a greater degree of precision should be possible.

One of the greatest variables noted in the second study was in the methods of interpreting and reporting the actual results. Some laboratories reported their results as "to 900 p.p.m." when the data showed an equal number of passes and failures at this level and 850 p.p.m. was the highest hardness level at which the required end point was obtained in all trials. Thus, it would appear that the maximum water hardness should probably have been reported as 850 p.p.m. Also, in some instances, data was reported as the highest level at which the required end point was found even though the product failed more times than it passed at that level. Tables 2 and 3 have been corrected for such differences insofar as feasible. However, one factor contributing to the size of the standard deviation could not be corrected for. In a number of instances laboratories employed tests using hardness concentration ranges of 100 p.p.m. and in such cases it was impossible to determine if an intermediary concentration of hardness would or would not have been the maximum tolerance level in that particular laboratory.



TABLE 2. RESULTS ON UNKNOWN QUATERNARY SAMPLE A

Laboratory	No. of Tests	Max. water hardness in p.p.m. showing 99.999% kill of E. coli within 30 seconds	
		<u>Individuals</u>	<u>Laboratory Averages</u>
A	1	450	413 $\pm$ 54 $\frac{1}{\text{—}}$
	2	450	
	3	450	
	4	350	
	5	350	
	6	350	
	7	350	
	8	350	
B	1	400	408 $\pm$ 14
	2	400	
	3	400	
	4	400	
	5	450	
	6	400	
C	1	350	350 $\pm$ 0
	2	350	
	3	350	
	4	350	
D	1	350	375 $\pm$ 25
	2	350	
	3	400	
	4	400	
E	1	500	475 $\pm$ 25
	2	450	



TABLE 2. RESULTS ON UNKNOWN QUATERNARY SAMPLE A

Laboratory	No. of Tests	Max. water hardness in p.p.m. showing 99.999% kill of E. coli within 30 seconds	
		Individuals	Laboratory Averages
F	1	450	$450 \pm 0 \frac{2}{1}$
	2	450	
	3	450	
	4	450	
G	1	400	$400 \pm 0$
	2	400	
	3	400	
H	1	400	$430 \pm 38$
	2	500	
	3	450	
	4	450	
	5	350	
36		$404 \pm 39$	

1/ 400 p.p.m. not run. Possible end point at this level for all 350 values reported.

2/ Reported as the average of 4 determinations  $\pm$  factor not known.



TABLE 3. RESULTS ON UNKNOWN QUATERNARY SAMPLE B

Laboratory	No. of Tests	Max. water hardness in p.p.m. showing 99.999% kill of E. coli within 30 seconds	
		Individual	Laboratory Averages
A	1	650	$650 \pm 0 \frac{1}{2}$
	2	650	
	3	650	
	4	650	
	5	650	
	6	650	
	7	650	
	8	650	
B	1	850	$808 \pm 25$
	2	800	
	3	850	
	4	800	
	5	800	
	6	750	
C	1	750	$738 \pm 19$
	2	700	
	3	750	
	4	750	
D	1	700	$713 \pm 19$
	2	700	
	3	700	
	4	750	
E	1	1000	$950 \pm 50$
	2	900	
F	1	750	$750 \pm ? \frac{2}{2}$
	2	750	
	3	750	
	4	750	



TABLE 3. RESULTS ON UNKNOWN QUATERNARY SAMPLE B (Continued)

Laboratory	No. of Tests	Max. water hardness in p.p.m. showing 99.999% kill of E. coli within 30 seconds	
		Individual	Laboratory Averages
G	1	800	800 $\pm$ 0
	2	800	
	3	800	
H	1	900	775 $\pm$ 125
	2	900	
	3	650	
	4	650	
Total		735 $\pm$ 65	

1/ Eight failures at 750 p.p.m. reported but not run at 700, latter figure probable end point.

2/ Reported as average of 4 separate determinations  $\pm$  factor not known.



As a part of the second study, duplicate samples of synthetic test waters were made up according to the procedure forwarded to all collaborators at varying levels of hardness and titrations made by the EDTA Method <sup>5/</sup> to determine the actual hardness levels obtained. These results are given in Table 4. It should be pointed out that more precise standardizations are possible by standardizing a large volume at a high level of hardness according to titration with subsequent dilution to the level desired employing boiled distilled water. For routine work, however, the rapid dilution procedure employed with the solution reported in Table 4 appears to be sufficiently precise.

TABLE 4. VARIATIONS IN SYNTHETIC HARD WATERS

Calculated levels of hardness	Actual hardness levels determined by titrations	
	Laboratory	Laboratory
	1	2
p.p.m	p.p.m.	p.p.m.
250	258	255
	251	250
350	345	350
	345	360
450	446	445
	440	450
650	669	650
	642	645
750	761	770
	781	760
850	891	845
	905	865



In the revised instructions sent out in the second study, it was specified that the stock culture of E. coli should be carried on A.O.A.C. Nutrient Agar rather than the Difco Agar listed by Chambers. One collaborator made comparative studies employing a stock culture carried on Difco Agar and one carried on A.O.A.C. Nutrient Agar. This study revealed no substantial differences in results. The actual data is summarized in Table 5.



TABLE 5. COMPARISON OF RESULTS OBTAINED USING STOCK CULTURES CARRIED ON A.O.A.C. NUTRIENT AGAR AND ON DIFCO NUTRIENT AGAR.

Water Hardness p.p.m.	Test No.	A.O.A.C. Nutrient Agar Per cent survival in 30 secs.	Difco Nutrient Agar Per cent survival in 30 secs.
250	1	< 0.0001	< 0.0001
	2	0	< 0.0001
	3	< 0.0001	< 0.0001
	4	0	0
350	1	< 0.0001	< 0.0001
	2	0.0007	< 0.0001
	3	< 0.0001	0.0001
	4	< 0.0001	< 0.0001
450	1	0.0012	0.0008
	2	0.0010	0.0010
	3	0.0009	0.0021
	4	0.0008	0.0022
650	1	< 0.0001	< 0.0001
	2	< 0.0001	< 0.0001
	3	< 0.0001	0.0006
	4	< 0.0001	0.0004
750	1	0.0014	0.0011
	2	0.0030	0.0011
	3	0.0093	0.0102
	4	0.0227	0.0048
850	1	0.0160	0.0111
	2	0.0154	0.0140
	3	0.0529	0.1207
	4	0.0510	0.1073



In the 1956 Associate Referee Report <sup>6/</sup> results were presented which indicated that the 30 second end point in the Chambers Method with hypochlorite was at the 50 p.p.m. available chlorine level. Extensive tests with commercial acid iodine preparations show that the end point in this method is often achieved when the pH is below 4.0 at a concentration providing 6.25 p.p.m. of titratable iodine. Since the lowest concentration with hypochlorite accepted as a starting solution is 100 p.p.m. of available chlorine and the lowest concentration of iodine accepted as a starting solution is 12.5 p.p.m. of titratable iodine, it would appear reasonable to multiply the lowest effective concentration at the 30 second interval in the method by 2 to determine the lowest concentration of germicide which should be recommended for use. This brings up a number of questions relative to acceptances with quaternary ammonium preparations.

A third collaborative study was initiated to determine the minimum concentrations of 2 quaternary ammonium preparations which would give the required 99.999% kill in the Chambers procedure at a water hardness level of 250 p.p.m. Sample A in this study contained a quaternary which in preliminary trials did not give the required end point at a 100 p.p.m. quaternary level in water of 250 p.p.m. of hardness but sample B did apparently give the required end point at this level.

The collaborative results from 3 laboratories are given in Table 6. The results reported by laboratories 2 and 3 would indicate that a factor of 2 times the minimum concentration giving the required 30 second end point



in the Chambers Method could be relied upon to provide a safe working solution and also that this factor would probably not require extensive revisions in the currently accepted labels for such products. Laboratory 1, on the other hand, reported results which would indicate that such a factor might require in some instances at least higher starting concentrations of quaternaries than 200 p.p.m.

TABLE 6. RESULTS ON VARYING CONCENTRATIONS OF UNKNOWN QUATERNARIES  
IN WATER OF 250 P.P.M. OF HARDNESS

Unknown Quaternary	Conc. P.P.M. Q.A.C.	Laboratory 1		Laboratory 2		Laboratory 3	
		Percentage kill in		Percentage kill in		Percentage kill in	
		30 Sec.	2 Min.	30 Sec.	2 Min.	30 Sec.	2 Min.
A	150	TNTC <sup>1/</sup>	99.999	99.999	99.999	99.999	99.999
	200	99.985	99.997	99.999	99.999	99.999	99.999
	250	99.998	99.999	99.999	99.999	99.999	99.999
	300	99.999	99.999	99.999	99.999	99.999	99.999
B	100	TNTC	99.997	99.999	99.999	99.999	99.999
	125	99.999	99.999	99.999	99.999	99.999	99.999
	150	99.999	99.999	99.999	99.999	99.999	99.999

<sup>1/</sup> TNTC - Colonies too numerous to count.  
No calculations made.



In connection with this particular study, one collaborator submitted data on a study of a quaternary ammonium detergent-sanitizer formulation wherein tests were made by the Chambers Method on the cleaning solution before and after actual use on a farm dairy. In this study titrations were also made to determine the concentrations of quaternaries using the method Furlong and Elliker.<sup>7/</sup> Some of these results have been listed in Table 7. These indicate losses of quaternary in use due to combinations with organic matter or otherwise on the order of 30-35%. They also indicate that residual concentrations of less than 130 p.p.m. of quaternary carrying organic residues may not have the minimum activity required in the Chambers procedure, under all conditions. These results are interpreted as added evidence indicating the necessity of a safety factor for interpreting results obtained by the Chambers Method with quaternary ammonium preparations of at least 60%. It is quite obvious from this data that the 100% factor found applicable for chlorine and iodine type products would be adequate with quaternary ammonium preparations insofar as effectiveness is concerned. It should be acknowledged, however, that the necessity for a safety factor of this magnitude for quaternaries is not actually demonstrated in this instance.



TABLE 7. RESIDUAL CONCENTRATIONS OF QUATERNARY AMMONIUM GERMICIDE IN THE CHAMBERS PROCEDURE IN FIELD STUDIES ON MILKING EQUIPMENT\*

Period of Use	P.P.M. Q.A.C. by titration	Tests by Chambers Method		
		Contact Period	No. of Colonies Surviving	Per cent Reduction
Before	186	30 Sec.	0	100.0
After	126	"	0	100.0
Before	158	30 Sec.	192	99.999
After	126	"	TNTC	99.9-
Before	192	30 Sec.	0	100.0
After	120	"	TNTC	99.9-
Before	212	30 Sec.	2	99.999
After	146	"	13	99.999
Before	210	30 Sec.	0	100.0
After	152	"	0	100.0
Before	196	30 Sec.	0	100.0
After	162	"	0	100.0
Before	212	30 Sec.	10	99.999
After	168	"	0	100.0

\* Data submitted by Dr. M. Speck, Department of Animal Industry, North Carolina State College, Raleigh, North Carolina.



If a factor of 2 times the minimum effective concentration in the Chambers procedure at 30 seconds is applied to determine the minimum concentration which can be recommended in use how can this be reconciled with the current application of the method in determining maximum hard water tolerances at the recommended concentrations?

Since there is a direct relation between quaternary concentration and water hardness, discrepancies cannot be avoided here unless the basis for determining the minimum effective concentration employing the safety factor indicated is tied to results obtained in distilled water and the maximum water hardness tolerance subsequently determined on the concentration determined by multiplying this value by 2; or, the current practice of determining maximum hard water tolerances based on the recommended use concentration abandoned in favor of a complete hard water-quaternary concentration activity curve determined by the Chambers Method. The latter alternative might be considered the most desirable in some quarters and should not be prohibited. However, the former can be more simply applied in the routine testing of commercial samples and would have to be adopted as the primary basis of current evaluations.

#### RECOMMENDATIONS

It is recommended that the Chambers modification of the Weber and Black Method be accepted as outlined below on a First-Action basis.

It has been suggested that samples A & B in the second study be accepted as official reference compounds for use in controlled tests when the method is applied to unknown quaternaries or samples of commercially distributed products. This suggestion has considerable merit and should be



given further study. Before such an action could be taken, it will be necessary to work out in detail such factors as standardization and identification of the reference material and methods of distribution to testing laboratories.

#### Germicidal Sanitizers and Detergent-Sanitizers.

##### (First Action)

(Suitable for use in determining the minimum concentrations of germicide which can be permitted in use and when multiplied by the factor 2 the minimum recommended starting concentration; also, the maximum water hardness tolerances for recommended concentrations.)

#### 1. Test Culture Media:

- (a) (1) Nutrient Agar - Boil 3 g. beef extract, 5 g. peptone (Bacto or equivalent) (special grades not to be used), 15 g. agar (use for daily transfer of test culture). (2) Nutrient Agar - Boil 3 g. beef extract, 5 g. peptone (Bacto or equivalent) (special grades not to be used), 30 g. agar, (use for growing test culture in French square bottles). (3) Nutrient Agar (A.O.A.C.) - Boil 5 g. beef extract (Difco), 5 g. NaCl, 10 g. Armour peptone, 15 g. agar, in 1 liter of distilled water, adjust to pH 7.2 - 7.4, (use for preparing stock culture slants).

#### (b) Subculture Media:

- (1) Use Tryptone glucose extract agar (Difco) plus 25 ml. of stock neutralizer per liter of agar. (2) Tryptone glucose extract agar (Difco).



2. Neutralizer Stock:

(a) (1) 40 g. Azolectin, 280 ml. Tween-80, 1.25 ml. phos. buffer, distilled water to 1 liter adjust to pH 7.2. Dispense in 100 ml. amounts and sterilize at 15 lbs. for 20 minutes.

(b) Neutralizer Blanks: (for 200 ppm Q.A.C. or less)

(1) Neutralizer stock 100 ml. M/4 phos. buffer pH 7.2, 25 ml. distilled water 1675 ml. Dispense in 150 x 20 test tubes 9 ml. per tube. Sterilize at 15 lbs. for 20 minutes.

3. M/4 Phosphate Buffer Stock:

Dissolve 34.0 g. potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$  in 500 ml. distilled water, adjust to pH 7.2 with 1 M NaOH and make up to 1 liter.)

4. Phosphate Buffer Dilution Blanks:

(1) Add 1.25 M/4 Phosphate Buffer stock to 1 liter distilled water, make up 99 ml. blanks.

5. Test Organisms:

(1) Use Escherichia coli ATCC 11229 or (2) Micrococcus pyogenes var. aureus FDA 209. Other species may be used for special purposes. Maintain stock culture on nutrient agar A.O.A.C.

6. Apparatus:

(1) Glassware - (a) Erlenmeyer flasks, wide mouth, 250 ml., (b) 100 ml. graduate, (c) Pipettes (milk type), (d) Test tubes 150 x 20. The above should be washed in strong, fresh cleaning solution, fill and drain at least 3 times. Sterilize for at least 2 hours at 180°C. in hot air oven.



- (e) Petri dishes - sterile
  - (f) French square bottles 175 ml. (borosilicate).
- (2) Water Bath - Appropriate water bath at 25°C.

7. Preparation of culture suspension:

From the stock culture make daily transfers for 3 successive days to nutrient agar slants 1, a, (1) incubating the transfers in 20-24 hours at 35°C. Do not continue to use a culture more than 30 days without starting anew from stock culture. If only a daily transfer has been missed, repeat with 4 daily transfers. Inoculate into 175 ml. Pyrex French square bottle (borosilicate glass) containing 20 ml. of nutrient agar 1,a,2, and allowed to solidify with bottle resting in a horizontal position. Culture bottles are inoculated by washing the growth from an agar slant into 99 ml. phosphate buffer dilution blank and adding 2 ml. of this suspension to each culture bottle which is tilted back and forth to distribute the suspension after which excess liquid is drained off. Incubation is for 18-24 hours at 35°C. agar side down. Culture is removed from the agar surface of 4 or more culture bottles using 3 ml. of phosphate buffered dilution water with the aid of glass beads to suspend the growth from each bottle. Suspension so prepared is filtered through No. 2 Whatman paper and collected in a sterile tube. Filtration may be hastened by rubbing the filter paper gently with a sterile policeman. This suspension is then standardized by dilution with sterile dilution water phosphate buffer using a Lumetron Colorimeter according to the following table to give an average of 10 billion organisms per ml., or a McFarland Nephelometer.



### Lumetron Colorimeter

	FILTERS							Average Bact. Count*
	370 Mu	420 Mu	490 Mu	530 Mu	550 Mu	580 Mu	650 Mu	
Percent	7.0	4.0	6.0	6.0	6.0	7.0	8.0	13.0
of	8.0	5.0	7.0	7.0	7.0	8.0	9.0	11.5
Light	9.0	6.0	8.0	8.0	8.0	9.0	10.0	10.2
Trans-	10.0	7.0	9.0	9.0	9.0	11.0	11.0	8.6
mission	11.0	8.0	10.0	10.0	10.0	12.0	13.0	7.7
	13.0	9.0	12.0	12.0	12.0	13.0	15.0	6.7

\* Bacteria count in billions/ml.

If the Lumetron is employed, the suspension is diluted in a sterile Lumetron tube.

### McFarland Nephelometer

(Barium Sulfate Standards)

Standard No.	2% Stock Soln. BaCl <sub>2</sub> ml.	1% Stock Soln. H <sub>2</sub> SP <sub>1</sub> ml.	Av. Bact. Count*
1	4.0	96.0	5.0
2	5.0	95.0	7.5
3	6.0	94.0	8.5
4	7.0	93.0	10.0
5	8.0	92.0	12.0
6	10.0	90.0	13.5
7	12.0	88.0	15.0

\* Bacteria count in billions/ml.



Select 7 tubes of the same internal diameter as that containing the test culture suspension. Put 10 ml. of each suspension of barium sulfate in each tube and seal up the tube. Best standardizations are obtained by diluting the suspension to correspond to the No. 4 standard.

8. Synthetic Hard Water:

(1) Two stock solutions are prepared. One, solution A, is made by dissolving 31.74 g of  $MgCl_2$  and 73.99 g  $CaCl_2$  in boiled distilled water and adjusting to 1 liter volume. The other solution B is made by dissolving 56.03 g  $NaHCO_3$  in boiled distilled water and adjusting to 1 liter volume. Solution A may be heat sterilized. Solution B cannot be heat sterilized, but it can be sterilized by filtration. The required amount of solution A is added to a sterile one liter flask and at least 600 mls. of sterile distilled water added. Then, 4 mls. of solution B is added and the volume brought to 1 liter with sterile distilled water. Each ml. of solution A used will give a water equivalent of approximately 100 p.p.m. of hardness calculated as  $CaCO_3$  using the formula:

$$\text{Total hardness as p.p.m. of } CaCO_3 = 2.495 \times \text{p.p.m. of } Ca + 4.115 \times \text{p.p.m. of } Mg.$$

The pH of all test waters up to 2,000 p.p.m. of hardness should fall between 7.6 and 8.0. Chemicals of reagent grade should be employed. If the hydrates of  $MgCl_2$  are used, substitutions should be made on an equimolar basis, respectively. All synthetic hard waters prepared for use by this procedure should be checked chemically for hardness at the time the tests are made, employing a procedure or procedures described



in the 10th Edition Standard Methods for the Examination of Water, Sewage and Industrial Wastes.

9. Performance of Test:

- (a) Measure 99 ml. of test water, containing bactericide at the concentration to be tested, into chemically clean, sterile, 250 ml. wide mouth Erlenmeyer flasks and place in constant temperature bath until it becomes stabilized at 25°C. or at least 20 minutes. Prepare duplicate flasks for each germicide to be tested. Also, prepare a similar flask, containing 99 ml. of sterile phosphate buffer dilution water as an "initial numbers" control.
- (b) Add 1 ml. of culture suspension to each test flask as follows: whirl flask, stopping just before suspension is added, creating sufficient residual motion of liquid to prevent pooling of suspension at point of contact with test water. Add suspension midway between the center and edge of the liquid surface with tip of pipette slightly immersed in test solution. Avoid touching pipette to neck or side of test flask during addition of suspension. Transfer 1 ml. portions to neutralizer blanks exactly 30 seconds and 60 seconds after addition of suspension. Mix well immediately after transfer. For a numbers control transfer, add 1 ml. of culture suspension to 99 mls. of sterile phosphate buffer dilution water in the same manner. In the case of the "numbers control," plants need only be made immediately after adding and thorough mixing not longer than 30 seconds. In the performance of the test, it is advantageous to use milk pipettes for adding culture and withdrawing samples.



- (c) Plate from neutralizer tube to agar. In the case of the quaternaries, use 1,b,(1). With the numbers control, use 1,b,(2). Where 1/10 ml. portions are planted, a 1-ml. pipette graduated at 1/10 ml. intervals is suggested. For necessary dilutions to give countable plates, use phosphate buffer dilution water (4). For the numbers control, the following dilution procedure is suggested:

1 ml. of exposed culture (1 ml. culture suspension transferred to 99 mls. phos. buffer in water bath) to 99 mls. phosphate buffer, dilution A. Shake thoroughly, and transfer 1 ml. dilution A to 99 mls. phosphate buffer, dilution B. Shake thoroughly, and transfer 1 ml. dilution A to 99 mls. phosphate buffer, dilution C. Shake thoroughly, and transfer 4 1 mls. and 4 0.1 ml. aliquots to individual sterile petri-dishes. For the test samples, the following dilution procedure should be sufficient:

1 ml. of exposed culture (1 ml. culture suspension + 99 mls. medicant) into 9 mls. of neutralizer 2.b.(1). Shake, and transfer 4 1 ml. and 4 0.1 ml. aliquots to individual sterile petri-dishes. For the numbers control, use Tryptone glucose extract agar 1.b.(2), and for the tests with quaternaries use Tryptone glucose extract agar, plus neutralizer 1.b.(1). Cool agar to solidify, and then invert and place in incubator.

- (d) Incubate plates in an inverted position at 35°C. for 48 hours before counting.



10. Results:

Results, to be considered effective must meet the standard effectiveness specified in the Chambers Method; that is, 99.999 percent reduction in the count of the number of organisms within 30 seconds. Report results according to actual count and percentage reduction over numbers control. The counts on the numbers control for the germicide test mixture should fall between 75 and 125 million per ml. for the percentage reductions to be considered valid.

11. Sterility Controls:

- (a) Neutralizer - plant 1 ml. from a previously unopened tube of neutralizer.
- (b) Each type of water used - 1 ml. quantity per plate.
- (c) Sterile distilled water - 1 ml. per plate.
- (d) After counting plates, confirm that surviving organisms were E. coli by transfer to brilliant green bile broth fermentation tubes or lactose broth and E.M.B. agar. Confirmation by straining is suggested with M. pyogenes var. aureus as the test organism.

12. Resistance to Phenol of Test Cultures:

- (a) It is recommended that the resistance to phenol be determined at least every three months by the A.O.A.C. Method

13. Unknown Samples:

The percentage composition given on the label or as determined should be used as a guide to the sample weight required for the volume of water used. From this stock dilution, 1 ml. can be transferred into 99 mls. of test water to give a 200-p.p.m. solution. In making this transfer, the 1 ml. pipette should be filled and allowed to drain back into the stock solution, and then refilled to correct



for adsorption on the glass. After mixing, 1 ml. can be discarded to provide the 99 ml. of test water 9.a.

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